Cytogenetic evaluation of tannery workers in the city of Teresina, northeastern Brazil

F.P.M. do Amaral[1], E.J.L.S. do Amaral[1], M.P.M. do Amaral[1], L.S. Lopes[1], S.I. Lemos[1], R.V.G. Santos[1], T.V.S. Chaves[2], M.E.A. de Moraes[3] and M.O.A. de Moraes Filho[3]

[1]Laboratório de Biologia Molecular e Estudo de Injúrias Biológicas, Faculdade de Ciências Médicas, Universidade Estadual do Piauí, Teresina, PI, Brasil
[2]Centro de Referência em Saúde do Trabalhador, Secretaria do Estado do Piauí, Teresina, PI, Brasil
[3]Laboratório de Oncologia Experimental e Unidade de Farmacologia Clínica, Universidade Federal do Ceará, Fortaleza, CE, Brasil

Corresponding author: F.P.M. do Amaral
E-mail: fabricio34amaral@gmail.com

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ABSTRACT. Assessments of chromosomal integrity and structure enable the prevention of diseases associated with the work environment, with the frequencies of chromosomal aberrations and micronuclei often being used as markers in biomonitoring. Owing to their routine manipulation of potentially toxic chemicals, tannery workers as a group merit a more thorough evaluation and discussion. This study investigated chromosomal damage in 30 workers from a tannery in the city of Teresina, the state capital of Piauí, northeast Brazil, and a control group consisting of 30 employees from a nearby accounting firm. The frequencies of chromosomal aberrations (CAs) and binucleated cell micronuclei (MN) were assessed as a measure of damage. Means were compared using the Student *t*-test and ANOVA-Dunnett test. Our results indicated a higher number of CAs in exposed individuals compared to the control group, including dicentric ($P < 0.0001$) and tricentric chromosomes ($P < 0.001$), and those in ring

(P < 0.0001) and acentric ring forms (P < 0.001). Assessment of MN frequency demonstrated a similar trend (exposed *vs* control, P < 0.0001). It was concluded that the tannery workers in this study exhibited a higher incidence of genetic damage than comparable unexposed individuals. However, further research on this subject is needed, particularly in regard to potentially clastogenic agents used in the tanning process.

Key words: Tannery workers; Chromosomal aberrations; Micronuclei

INTRODUCTION

Tannery workers are routinely exposed to a large number of chemical agents. For this reason, various studies have been carried out to evaluate their health. Asthma (Shahzad et al., 2006), stomach disorders (Rastogi et al., 2008), and increased cancer incidence (Veyalkin and Gerein, 2006) have all been identified in epidemiological investigations, and have been associated with exposure to the metal chromium (Rastogi et al., 2008). In addition, genetic studies have obtained evidence that an environmental agent is responsible for altering cellular integrity in such individuals, and possibly contributing to the onset of these diseases. Research such as that conducted by Hamamy et al. (1987), Hilali et al. (2008), Monteiro Neto et al. (2010), and Balachandar et al. (2010) has addressed the issue of chromosomal aberrations (CAs) in tannery workers. Although initial results obtained by Hamamy et al. (1987) indicated an absence of genetic alterations in this group, later studies produced contradictory results, demonstrating that further investigation was required. In Brazil, Monteiro Neto et al. (2010) evaluated 10 employees of a tannery in the city of Franca in São Paulo State, southeast Brazil. Their results revealed a higher frequency of CAs in individuals exposed to tannery chemicals. This finding corroborates those found in the literature following the Hamamy et al. (1987) investigation. In order to contribute to the body of knowledge relating to this issue, a cytogenetic evaluation of tannery workers exposed to chemical hazards in the city of Teresina, northeast Brazil, was carried out.

MATERIAL AND METHODS

Subjects

Our study included 30 tannery employees and 30 unexposed individuals employed at a local accounting office, for a total N = 60. The former participated directly in the tanning process, working on the separation area, wet blue, semi-finished, and production stages, with no specific role. Questionnaires were used to collect personal (age, working hours, health details, etc.) and lifestyle data (eating habits, alcohol and tobacco consumption, etc.) from both groups.

Study site

Teresina, state capital of Piauí (Figure 1), is located inland in a region known as the mid-north of Brazil. According to the Brazilian Institute of Geography and Statistics (IBGE, 2014), the estimated population is 840,600, covering 1,391,981 km^2, and has a human development index, 0.751. The city is a reference for its high standard of healthcare. Teresina has a functioning tannery,

however, according to data from the Tanning Industry Center of Brazil (CIC, 2013), Piauí was ranked 12th in Brazil for leather exports between the years 2011 and 2013.

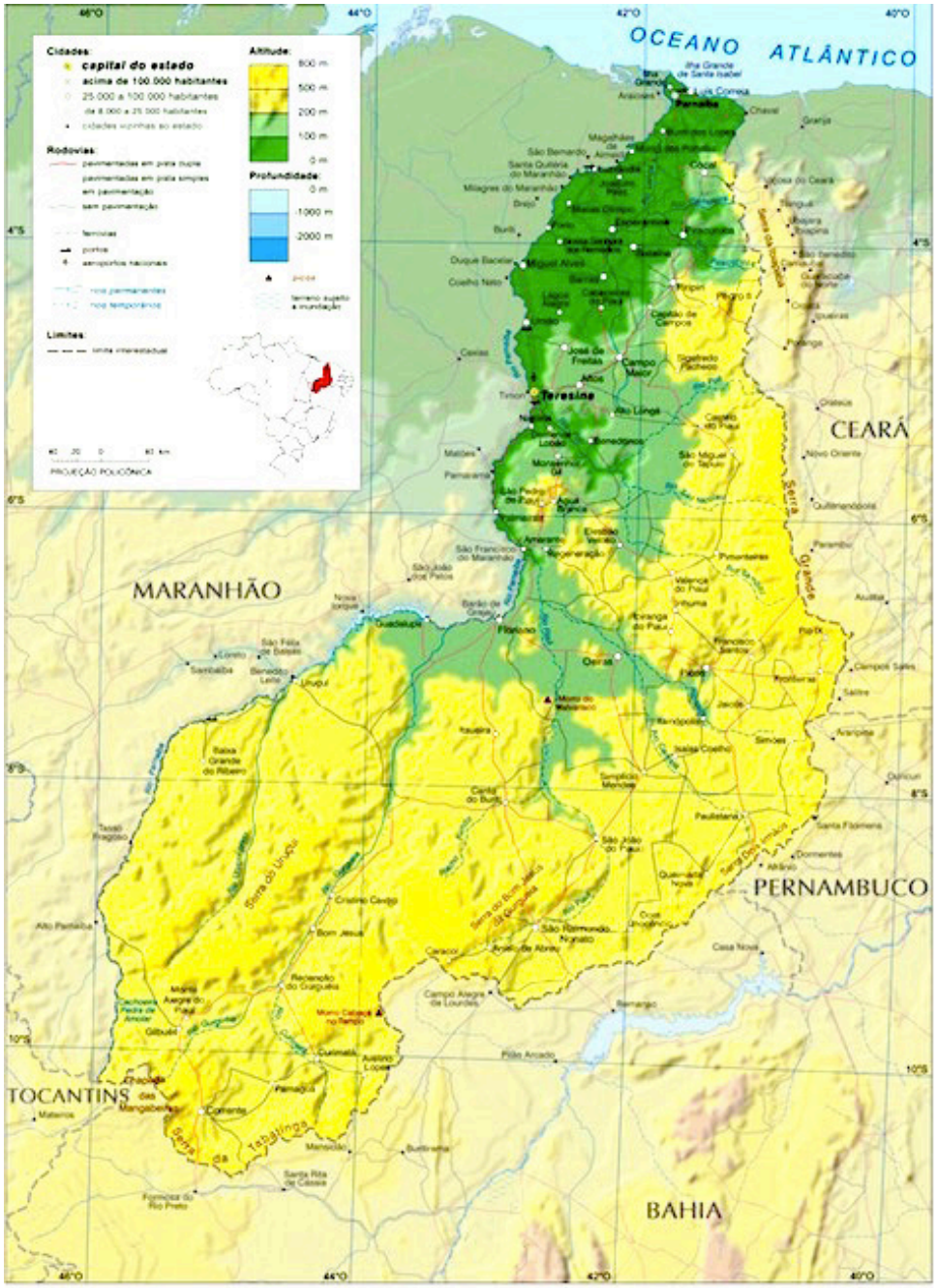

Figure 1. Piauí State with its capital, Teresina, highlighted.

The tannery is located at 380 Avenue Poty Velho, on the outskirts of the capital in the neighborhood of Santa Maria da Codipi. At the time of study, it routinely processed 1000 pieces of leather per month. Working hours were Monday to Saturday, from 07:00 to 18:00. Raw material in the form of animal hide came from the states of Ceará, Maranhão, and Piauí. There were a total 65 employees, 30 of whom were responsible for the management of the tanning process.

Sample collection and lymphocyte culture

Following completion of the questionnaire, 5 mL peripheral blood was collected from each study participant using sterile, heparinized syringes. Blood samples were then sent to the Laboratory of Molecular Biology and Biological Injuries Studies (LABMINBIO), Faculty of Medical Sciences, State University of Piauí (FACIME/UESPI), and the Cytogenetics Laboratory of the Federal University of Piauí (UFPI).

The lymphocyte culture protocol for the analysis of CAs and micronuclei (MN) followed the classical criteria of Moorhead et al. (1960) and Preston et al. (1987), with some modifications. Eighteen drops of leukocyte suspension were added to 5 mL RPMI 1640 medium supplemented with 15% fetal bovine serum, 1% penicillin and streptomycin mixture, 1% L-glutamine, and 1% phytohemagglutinin (to stimulate cell division). The tests were carried out in triplicate, i.e. three flasks each were used for CA and MN assessments. The cultures were kept in a CO_2 incubator (IG 150; Jouan, Waltham, MA, USA) at 37°C for 72 h.

Study of CAs

Two hours before the incubation was complete, 16 µg/mL colchicine (Sigma, St. Louis, MO, USA) was added to the flasks used for CA assessment. After 72 h, the lymphocyte culture was transferred to sterile 10-mL Falcon tubes (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 1000 rpm for 5 min, following which, hypotonic solution consisting 0.075 M KCl was added. Slides were fixed in a 3:1 methanol:acetic acid (v/v) mixture and stained with 5% Giemsa solution, pH 6.8, before being viewed under an optical microscope (CX41; Olympus, Tokyo, Japan), using oil immersion and 100x magnification. One hundred metaphases per slide per individual were analyzed. Dicentric and tricentric chromosomes, those in ring and acentric ring forms, chromosomal fragments, and terminal and interstitial deletions were counted. Our analysis was based on Savage's classification (1976). The metaphase index (MI) was calculated by multiplying the number of metaphases by 100 and dividing this figure by 300. Similarly, CA frequency was calculated by multiplying the number of CAs by 100 and dividing the resulting value by 300.

Cytokinesis-block MN test

The evaluation of MN in binucleated cells followed the protocol established by Pastor et al. (2003) and Fenech (2007), with some adjustments. Approximately 6 µg/mL cytochalasin B in dimethyl sulfoxide (Sigma) was added to cultures after 44 h incubation. Seventy-two hours later, cells were centrifuged at 800 rpm for 8 min, then rinsed in RPMI 1640 medium, and treated with 4°C 0.075 M KCl hypotonic solution for 3 min. This procedure was repeated three times. The cells were then centrifuged again and fixed in a 3:1 methanol:acetic acid (v/v) solution. This stage was repeated twice, taking care to manipulate the samples gently. Finally, cells were resuspended and

placed on clean plates, before being incubated with 10% Giemsa stain in phosphate buffer, pH 6.8, for 10 min. Analysis was carried out under the abovementioned light microscope using 100x magnification. Approximately 1000 binucleated cells with preserved cytoplasms were evaluated for MN frequency according to the criteria of Fenech (2007).

Statistical analysis

Statistical analysis was carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 4.0 (GraphPad Software, La Jolla, CA, USA). Simple frequency analysis and comparison of means using the Student *t*-test, and association analysis using the chi-square test, were carried out. For assessment of CAs, we used ANOVAs followed by Dunnett's tests. P values less than 0.05 were considered statistically significant.

Ethical issues

The current research protocol was approved by the Ethics Committee of the Federal University of Ceará (UFC/CE; number 195/08). Informed consent statements were signed by all participants and confidentiality was ensured.

RESULTS

Characteristics of the study population are shown in Table 1. The groups were paired and no statistically significant differences regarding mean age and working hours were observed. Table 2 shows the MI values and frequencies of the main CA types found in lymphocytes of exposed and unexposed individuals. The MI did not significantly differ between the two groups, however, the total number and frequency of CAs was significantly higher in tannery workers. The principal types of CA found in exposed individuals were dicentric and tricentric chromosomes, and those in ring and acentric ring forms. Terminal deletions and chromosomal fragments were not frequently observed. Table 3 compares MN number and frequency between exposed and unexposed workers, demonstrating that both of these measures were significantly higher in tannery employees.

No significant differences regarding CAs and MN were found between smokers and non-smokers or between alcohol consumers and non-consumers within the tannery worker group (data not shown).

Table 1. Characteristics of exposed and unexposed groups obtained from personal health questionnaires.

	Exposed			Not exposed		
	Total	Smokers	Non-smokers	Total	Smokers	Non-smokers
N	30	11 (36.66%)	19 (63.33%)	30	4 (13.33%)	26 (86.66%)
Alcohol drinkers	26 (86.66%)	11/26	15/26	28 (93.33%)	4/28	24/28
Age*	34.42 ± 9.61 (18-65)	40.3 ± 10.72 (32-65)	32.13 ± 8.81 (18-37)	32.40 ± 8.10 (20-51)	46.00 ± 6.31 (43-51)	30.52 ± 10.11 (20-38)
Hours worked/week*	44.51 ± 0.96 (44-50)	44.51 ± 0.96 (44-50)	44.51 ± 0.96 (44-50)	44.19 ± 0.93 (44-50)	44.19 ± 0.93 (44-50)	44.19 ± 0.93 (44-50)
Exposure time	8.21 ± 5.75	9.3 ± 7.32	5.32 ± 3.45	0	0	0

*Data are reported as mean values with standard deviations and ranges. A P value greater than 0.05 indicated no significant differences between the tannery workers and the control group in terms of weekly working hours and age, based on the chi-square test.

Table 2. Major chromosomal aberrations (based on 100 metaphases per individual) found in workers exposed to the tanning process and unexposed controls.

Group	No. of cells	MI %	Type of CA							Total CA	CA %
			Dicentric	Tricentric	Ring	Acentric ring	Fragments	Terminal del.	Interstitial del.		
Exposed N = 30	376.2 ± 106.0 (218-502)	109.2 ± 14.6 (75-141)	53.9 ± 23.3*** (0-99)	2.1 ± 0.6** (0-10)	4.9 ± 4.0*** (0-15)	2.35 ± 1.45** (0-26)	1.00 ± 0.50 (0-9)	1.9 ± 0.51 (0-8)	0.35 ± 0.1 (0-2)	16.3 ± 3.38*** (4-51)	8.01 ± 1.82*** (0.8-43.7)
Not exposed N = 30	322.6 ± 67.80 (195-432)	101.9 ± 14.8 (57-125)	5.4 ± 1.74 (0-6)	0.0 ± 0.0 (0-0)	0.0 ± 0.0 (0-0)	0.05 ± 0.05 (0-1)	0.05 ± 0.05 (0-1)	0.10 ± 0.06 (0-1)	0.0 ± 0.0 (0-0)	0.60 ± 0.16 (0-2)	0.403 ± 0.09 (0-2.2)

Data are reported as mean values with standard deviations and ranges. A P-value greater than 0.05 indicated no significant difference between the two groups in terms of metaphase index. **P < 0.001 and ***P < 0.0001 compared to the control group, based on ANOVA followed by the Dunnett test. MI = metaphase index; CA = chromosomal aberration; del. = deletion.

Table 3. Number and frequency of micronuclei in binucleated lymphocytes in exposed and control groups.

Group	No. of MN per 1000 binucleated cells	MN frequency
Exposed N = 30	17.73 ± 9.44* (0-33)	0.886 ± 0.472* (0-1.65)
Not exposed N = 30	10.90 ± 7.092 (2-30)[b]	0.306 ± 0.202 (0-0.75)

Data are reported as mean values with standard deviations and ranges. *P < 0.05 for incidence of micronuclei in binucleated cells compared to that of the control group, based on the Student *t*-test. MN = micronuclei.

DISCUSSION

The relationship between CAs and neoplasia has been investigated since the 1960s (Bonassi et al., 2004). Cohort studies monitoring CAs in individuals over time and attempting to correlate them with oncogenesis have shown that environmental factors, such as occupational exposure, are not decisive in causing malignancies (Bonassi et al., 2004; Norppa et al., 2006). However, studies of tannery workers in various countries have found significant differences between the number of CAs in exposed individuals compared to unexposed controls (Hilali et al., 2008; Monteiro Neto et al., 2010). Other reports of alterations to genetic material, such as an increase in DNA lesions, have confirmed these results (Balachandar et al., 2010; Ambreen et al., 2014). In our study, dicentric, tricentric, and ring-shaped chromosomes were the most frequently observed aberrations (Figure 2). Ring chromosomes have not been detected in previous studies of tannery workers. These abnormal structures are found in many types of human neoplasia, and according to Bonassi et al. (2008), the appearance of dicentric and ring chromosomes is associated with an increased risk of carcinogenesis (with relative risks of 2.22 and 1.32 for ring and dicentric forms, respectively).

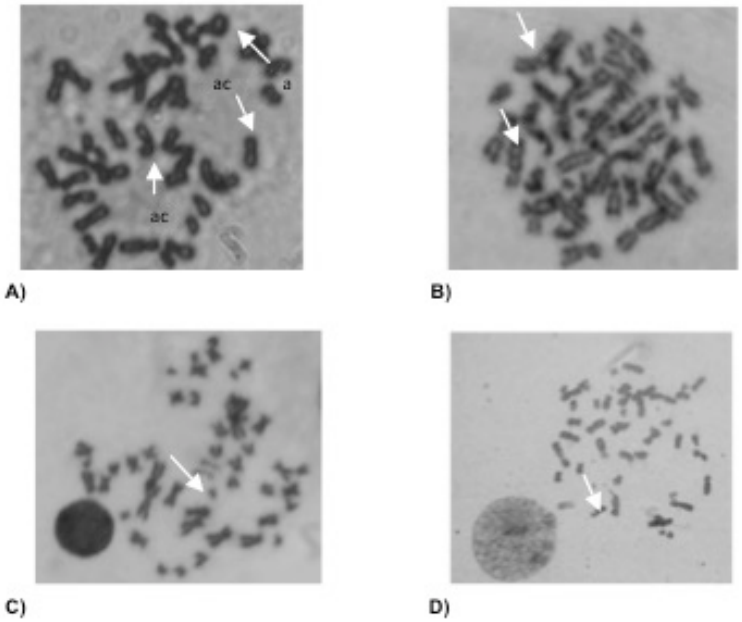

Figure 2. Examples of the chromosomal aberrations found in workers exposed to the tanning process. (**A**) Chromosomes in ring (a) and acentric ring (ac) forms; (**B**) dicentric and tricentric chromosomes; (**C**) terminal and interstitial deletions; and (**D**) chromatid-type aberrations.

In addition, the current study revealed that, as with CAs, the frequency of MN in binucleated cells was higher in exposed individuals. Increased prevalence of MN in tannery workers has already been documented in studies carried out in Portugal, Morocco, and India (Medeiros et al., 2003; Hilali et al., 2008; Balachandar et al., 2010). This finding confirms the clastogenicity evident from the CAs identified in this study.

A recent meta-analysis aiming to evaluate the role of MN as cancer biomarkers concluded that there exists a need to improve the design of studies relating to such research, since most investigations include several variables hindering the use of MN as reliable markers (Cardinale et al., 2012). However, according to Iarmarcovai et al. (2008), Bonassi et al. (2011), and Kirsch-Volders et al. (2014), the presence of MN is an important measure of chromosomal damage and is linked to environmental factors and lifestyle habits.

Several products used in the processing of animal hides may be implicated in the chromosomal damage found in this study. However, chromium in particular stands out as one of those potentially responsible. Hexavalent chromium's ability to interact with human DNA and promote the formation of adducts has been well established (Salnikow and Zhitkovich, 2008). The formation of DNA adducts creates instability in the replication fork, leading to chromosomal breaks (Kirsch-Volders et al., 2014). Moreover, several studies in which chromium levels have been measured in tannery workers have found large amounts in biological samples (Randall and Gibson, 1989; Stupar et al., 1999; Medeiros et al., 2003; Ambreen et al., 2014).

Our investigation did not assess chromium concentration in the individuals studied, therefore we cannot connect the genotoxicity of this metal to the results obtained. However, using these data, we can infer that tannery workers sustain a greater amount of genetic damage than unexposed individuals. Contact with an environmental factor in the workplace may contribute to the development of these alterations. This hypothesis reinforces the suspicion surrounding chromium and establishes the need for more thorough biomonitoring studies. This will enable a better understanding of the relationship between environmental exposure and genetic anomalies, and encourage the adoption of preventive measures against future damage.

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